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Transcription coupled nucleotide excision repair in *Escherichia coli* can be affected by changing the arginine at position 529 of the β subunit of RNA polymerase

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Abstract

The proposed mechanism for transcription coupled nucleotide excision repair (TCR) invokes RNA polymerase (RNAP) blocked at a DNA lesion as a signal to initiate repair. In *Escherichia coli*, TCR requires the interaction of RNAP with a transcription-repair coupling factor encoded by the *mfd* gene. The interaction between RNAP and Mfd depends upon amino acids 117, 118, and 119 of the β subunit of RNAP; changing any one of these to alanine diminishes the interaction [1]. Using direct assays for TCR, and the *lac* operon of *Escherichia coli* containing UV induced cyclobutane pyrimidine dimers (CPDs) as substrate, we have found that a change from arginine to cysteine at amino acid 529 of the β subunit of the RNAP inactivates TCR, but does not prevent the interaction of RNAP with Mfd. Our results suggest that this interaction may be necessary but not sufficient to facilitate TCR.

1. Introduction

Nucleotide excision repair (NER) removes many different kinds of lesions from DNA, including those resulting from exposure to UV. Two subpathways of NER have been described: global genome repair (GGR), which removes lesions from the genome overall; and transcription coupled repair (TCR), which removes lesions from the transcribed strand of active genes [2–4]. In *Escherichia coli* the product of the *mfd* gene is required for TCR [5–8]; *mfd* mutants do not exhibit TCR [9]. It has been proposed that, in addition to the Mfd protein, RNA polymerase (RNAP) is intimately involved in TCR, facilitating recognition of lesions in actively transcribed DNA strands [10], and interacting with Mfd which then recruits UvrA, which, together with UvrB and UvrC, is required for the initiation of NER (both TCR and GGR) [11–15]. Studies using RNAP from various sources have shown that cyclobutane pyrimidine dimers (CPDs), the prototype substrate for TCR in vivo, block transcription in vitro. The blocked RNAP occludes the lesion and interferes with its repair [5,16–20]. Mfd can dissociate RNAP from the lesion site allowing access to repair proteins [6–8]. The *E. coli* RNAP elongation complex consists of 5 subunits, α_2 , β , β' , and ω . Interaction with Mfd involves the N-terminal portion of the β subunit [1,7,8,21].

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RNA polymerase (RNAP) may stall on undamaged DNA templates when it encounters a pause sequence or a bound protein. Under appropriate conditions Mfd can interact with the β subunit of stalled RNAP and either terminate transcription or enable transcription to restart [7,21]. Results obtained with a yeast 2-hybrid system indicated that the binding site for Mfd lay within the first 142 amino acids of the N-terminal region of the β subunit terminal [21]. More recently, Smith and Savery [1] identified amino acids 117 (isoleucine), 118 (lysine), and 119 (glutamic acid) within this region as those required for the interaction. Substitution of any one of these with alanine reduced the interaction of RNAP with Mfd.

The *rpoB* gene codes for the β subunit of RNAP. Because of the potential importance of the β subunit for TCR, we analyzed TCR in *rpoB* mutants, specifically those resistant to the antibiotic rifampicin (Rif). In mutants in which the arginine at position 529 in the β protein had been changed to cysteine we observed a loss in the capacity for TCR. This response was unexpected because this part of the RpoB protein has not previously been implicated in interactions with Mfd, and it suggests that the ability to interact with Mfd is not the only property of RNAP required for TCR in vivo.

2. Materials and Methods

2.1. Bacteria

Table 1 lists the derivatives of *E. coli* K-12 used.

Table 2 lists the nucleotide and predicted amino acid changes in each strain.

2.2. Media

The minimal medium (MM) was Difco Minimal Broth Davis containing 0.4% glucose and 0.05% vitamin-free casamino acids. For plates, 1.5 % Difco Bacto agar was added. The complex medium was LB [22]. For LB plates, 1.2% Difco Bacto agar was added. L-broth [23] containing 1 g/l of glucose was used for preparing P1vir phage.

For thymine requiring strains, 10 μ g/ml of thymine was added to all media.

Rifampicin was used at a concentration of 100 μ g/ml, ampicillin at 30 μ g/ml, and tetracycline at 15 μ g/ml.

2.3. Transduction

Lysates of P1vir were prepared and transductions performed essentially as described by Miller [24]. To avoid isolating new *rpoB* mutants instead of the desired *rpoB* transductants, selection for tetracycline resistance (*btuB*::Tn10), not rifampicin resistance, was used. About 70% of *btuB*::Tn10 transductants acquire the linked *rpoB* allele of the donor strain [25]. Rifampicin resistance was used only to distinguish between transductants that had acquired the *rpoB* mutation and those that had not.

2.4. Irradiation

The UV source was an unfiltered 15 w germicidal bulb emitting primarily 254 nm. The incident dose rate was 0.6 J/m²/sec.

2.5. Assays for β -galactosidase

Enzyme activity was assayed with *o*-nitrophenyl- β -D-galactoside (ONPG) essentially as-described by Miller [22].

2.6. Repair assays

The frequencies of CPDs in individual strands of the *lac* operon were measured as previously described [10,26]. Exponentially growing cultures of *E. coli* in defined medium were irradiated and reincubated to allow repair to occur. Samples (5 ml) were removed from the culture at intervals, and mixed with an equal volume of ice cold NET (100 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl). The cells were pelleted by centrifugation and stored at -80°C . The DNA was subsequently purified on QIAGEN Genomic-tip columns, then treated with restriction enzymes (SacII and ApaI to produce a 6.6 kb fragment containing the entire *lac* operon), and analyzed by electrophoresis in alkaline agarose gels.

CPDs were detected with T4 endonuclease V (TEV), and CPD frequencies were calculated from the percentage of fragments that had no CPDs (the "0 class"), using the Poisson expression [26].

Because the results of individual assays were often variable, we typically performed each experiment at least twice, usually 3 times. Samples from each experiment were analyzed on 2 sets of duplicate alkaline agarose gels, giving a total of 4 gels for each experiment. After the DNA had been transferred from the gels to nylon membranes (Amersham), each membrane was hybridized with a ^{32}P -labelled strand-specific probe for the fragment of interest. The amount of radioactivity hybridized P-to the DNA representing the full length fragment of interest was measured using a phosphorimager (Bio-Rad GS-363 Molecular Imaging System) and associated software (Molecular Analyst, version 2.1). After obtaining the results from the first hybridization, we deprobed each membrane and then hybridized it with a probe for the other strand of the fragment. Typically one member of a pair of membranes was first hybridized with the probe for the transcribed strand (TS) and the other member of the pair was first hybridized with the probe to the nontranscribed strand (NTS). Results from each of the experiments were averaged so that for each data point on a graph, the CPD frequency was obtained from averaging 8 – 12 values. For statistical analysis and graphing we used Prism 4 (GraphPad Software, Inc.). Error bars on graphs represent the SEM.

2.7. DNA Sequencing

Mutations were verified using primers described previously [27,28] for amplifying and sequencing the region of the *rpoB* gene designated "cluster II" by Miller et al [27] (N.B. this terminology differs from that used by other investigators, e.g. [25,29]). Sequencing was done by Sequetech Corp. (Mountain View CA). Sequencher 4.6 (Gene Codes Corp., MI) was used to compare the sequences to the wild type listed in EcoCyc [30].

2.8. RNA polymerase displacement assays

Displacement of stalled wild-type and mutant elongation complexes by Mfd was analysed by electrophoretic mobility shift assays (EMSAs). Elongation complexes were formed on a 274 bp fragment of pSRT7A1 [31] that was amplified by PCR. Transcription initiating from the T7A1 promoter requires the incorporation of UTP at +2 and +21. Transcription was therefore initiated using the dinucleotide ApU and RNAP was stalled at +21 by the addition of 3'dUTP (TriLink Biotechnologies). Displacement assays were carried out as described [1] except that the nucleotide concentrations used were 100 μM ApU, 2 mM ATP, 10 μM GTP, 10 μM CTP and 10 μM 3'dUTP.

The plasmid encoding the His-tagged RNAP β R529C subunit is a derivative of pETLRpoBHis [1] constructed by site-directed mutagenesis.

Mfd protein and His-tagged wild-type and mutant RNAP holoenzyme were purified as described previously [1,32]

3. Results

To test for TCR, we measured repair of CPDs in the DNA of UV irradiated bacteria, comparing the rates of repair of the TS and the NTS of the *lac* operon. As previously described [10], TCR results in a strand bias, with CPDs being removed from the TS of the induced operon more rapidly than from the NTS. When the operon is not induced, both strands are repaired at the same rate as the NTS of the induced operon, which reflects the rate of GGR [33]. We tested 4 derivatives of MG1655 (HL961) for TCR. Of these, 2 (HL978 and HL979) showed little or no TCR (Fig. 1) while 2 (HL1080 and HL1082) showed relatively normal levels compared to HL961 (MG1655) (Fig. 2). We also found that repair of the transcribed strand of the *lac* operon in HL961 was not as rapid as in HL108 (SR108), the strain originally used by Mellon [10] (Fig. 3), and therefore the strand bias was less pronounced.

Our laboratory strains HL978 and HL979 were initially thought to correspond to strains CAG3008 and CAG3009, and to contain the *rpoB* alleles 3401 and 3402, respectively [25]. However, sequencing revealed that HL978 and HL979 contained the same allele, 3401 (Table 2), which causes a change of the arginine at position 529 in the β subunit to cysteine (R529C). This meant that only one amino acid change at position 529 of the RpoB protein was studied rather than the two we had anticipated. It was, however, reassuring that the repair properties of the two strains were essentially the same (Fig. 1).

Sequencing showed that HL1080 contained G instead of A at nucleotide 1552 resulting in a substitution of aspartic acid for asparagine at position 518 of the protein, while HL1082 contained a T instead of a C at nucleotide 1691 resulting in a substitution of leucine for proline at position 564. Neither of these changes affected TCR (Fig. 2).

All 4 of the strains tested for TCR are resistant to rifampicin, but of the amino acids affected by the mutations, only R529 interacts directly with the antibiotic. The other two, N518 and P564, are located near the rifampicin binding pocket, but they do not interact directly with the antibiotic [29].

To verify that the diminished capacity for TCR observed in HL979 was due to the *rpoB* mutation it carried, we transferred the mutation to HL108 by transduction. By selecting for tetracycline resistance we obtained *btuB*::Tn10 transductants that were either sensitive (wild type *rpoB* allele) or resistant (mutant *rpoB* allele) to rifampicin. We isolated one of each (HL1126 and HL1128, respectively). When these strains were tested, HL1126 exhibited robust TCR levels, while HL1128 showed no TCR (Fig. 4).

A possible explanation for low levels of TCR could be low levels of transcription of the gene being examined. This is particularly a concern in the case of strains with mutations like the one in HL978, HL979, and HL1128 that cause slow growth [34]. To verify that the *lac* operon was actively transcribed in the induced cultures, samples of the induced and noninduced cultures were assayed for β -galactosidase just prior to irradiation. Although measuring the protein product of a gene is not always a direct measure of transcription, in the case of *lacZ* induced by IPTG, increased β -galactosidase activity does reflect increased transcription. In all cases the induced cultures contained higher levels of β -galactosidase activity than the noninduced cultures (Table 3).

A second possibility for diminished TCR would be failure of the mutant β subunit to allow RNAP to interact with Mfd. Although R529 is not in the region of RpoB previously identified as being necessary for interaction with Mfd, we evaluated the possibility that this amino acid was important for interaction by measuring the effect of Mfd on the dissociation of stalled RNAP using purified proteins in vitro [1,32]. The results of these assays indicated that Mfd could interact normally with RNAP containing the mutant RpoB (Fig 5). Results obtained using

a reporter gene to assess dissociation of the mutant RNAP stalled by a "road block protein" in vivo [14,32] were consistent with this conclusion (data not shown). Based on these data we concluded that the lack of TCR in the mutant is not due to failure to interact with Mfd.

A third possibility for the effect of R529C on TCR might be instability of stalled transcription complexes. If RNAP containing the mutant β subunit rapidly dissociated from DNA when stalled it might be unable to act as a signal for the initiation of TCR. In in vitro assays, however, in the absence of Mfd, stalled transcription complexes containing RNAP β R529C showed no spontaneous dissociation within 30 minutes (Figure 5), suggesting that inherent instability of the mutant transcription complexes is not the cause of the absence of TCR.

4. Discussion

Our results indicate that changing the arginine residue at position 529 in the β subunit of RNAP can affect TCR of the *lac* operon in *E. coli*. The *rpoB* allele that changes the arginine to cysteine significantly reduced or eliminated TCR of the induced *lac* operon (Fig. 1 and Fig. 4), but did not appear to inhibit GGR as judged by the relatively normal rates of repair of the NTS, a valid indicator of GGR [33]. The defect in TCR was not due to lack of transcription of the gene examined. The production of β -galactosidase indicated that active RNAP complexes were traversing the *lac* operon immediately prior to irradiation (Table 3) and should have been available after irradiation to target TCR to damage in the operon. Because the UV dose used produced an average of about 1 CPD per strand of the operon, only 1 or 2 transcription complexes per operon would be needed for this.

In *E. coli* TCR requires the Mfd protein, which interacts with RNAP blocked at the site of a DNA lesion, and dissociates RNAP from the DNA, allowing repair proteins access to the lesion. It may also recruit repair proteins to the site, enhancing the rate of repair [5–8,11–15]. Previous studies have implicated amino acids in the first 142 residues of the β subunit of RNAP [21], and more specifically, amino acids 117, 118, and 119 [1], in the interaction of Mfd with RNAP. A change at amino acid 529 would thus not be expected to disrupt the interaction. Furthermore, R529 of the β subunit of the *E. coli* RNAP corresponds to R409 of the *Thermus aquaticus* protein, which interacts directly with rifampicin. The antibiotic "binds in a pocket of the RNAP β subunit deep within the DNA/RNA channel, but more than 12 Å away from the active site" [29]. By analogy with R409 of the *T. aquaticus* protein, R529 of the *E. coli* RpoB is likely to be exposed deep within this channel in a position that makes direct interactions with other proteins unlikely. As expected, we found that Mfd can dissociate stalled RNAP containing the R529C β subunit from DNA in vitro (Fig. 5) and in vivo (data not shown). In these experiments the RNAP was stalled by a chain terminating nucleotide (Fig 5) or by a "road block protein", rather than by a CPD; however, the fact that the mutant RNAP was able to interact with Mfd under these conditions indicates that it could also interact with Mfd when blocked at a CPD. At least in the case of eukaryotic RNA pol II, the conformation of the polymerase did not change when it was stalled at a CPD in vitro [35]. If this were also true for the *E. coli* RNAP, there should be essentially no difference in the conformation of RNAP stalled at any of these three kinds of sites.

From our results it is clear that there is at least one site in the β subunit of RNAP which is required for TCR in vivo and which is different from those previously identified as interacting with Mfd. Studies of other *rpoB* mutants may identify additional sites and help to clarify the precise requirements for TCR.

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Figure 1.

TCR in the *lac* operon of (A) HL978 and (B) HL979. Bacteria were grown with IPTG to induce transcription of the *lac* operon, or were grown without IPTG (noninduced). They were irradiated with a UV dose of 40 J/m^2 , and reincubated to allow repair to occur. Repair in a 6.6 kb DNA fragment containing the *lac* operon was measured. Error bars represent SEM. Symbols: open squares, TS of induced culture; filled squares, TS of uninduced culture; open triangles, NTS of induced culture; filled triangles, NTS of uninduced culture.

Figure 2.

TCR in the *lac* operon of (A) HL1080 and (B) HL1082. Bacteria were grown with IPTG to induce transcription of the *lac* operon, or were grown without IPTG (noninduced). They were irradiated with a UV dose of 40 J/m², and reincubated to allow repair to occur. Repair in a 6.6 kb DNA fragment containing the *lac* operon was measured. Error bars represent SEM.

Symbols: open squares, TS of induced culture; filled squares, TS of uninduced culture; open triangles, NTS of induced culture; filled triangles, NTS of uninduced culture.

Figure 3.

TCR in the *lac* operon of HL108 compared to HL961. Bacteria were grown with IPTG to induce transcription of the *lac* operon. They were irradiated with a UV dose of 40 J/m², and reincubated to allow repair to occur. Repair in a 6.6 kb DNA fragment containing the *lac* operon was measured. Error bars represent SEM. Symbols: squares, TS; triangles, NTS; open symbols, HL108; closed symbols, HL961.

Figure 4.

TCR in the *lac* operon of HL1126, and HL1128 (R529S). Bacteria were grown with IPTG to induce transcription of the *lac* operon. They were irradiated with a UV dose of 40 J/m², and reincubated to allow repair to occur. Repair in a 6.6 kb DNA fragment containing the *lac* operon was measured. Symbols: filled squares, TCR of HL1126; filled triangles, NTS of HL1126; open squares, TCR of HL1128; open triangles, NTS of HL1128.

Fig. 5. Displacement of RNAP βR529C by Mfd *in vitro*. (A) EMSA showing the displacement of RNAP βR529C by Mfd. Initiation complexes (IC) were formed by incubation of RNAP βR529C holoenzyme with a radio-labelled fragment of pSRT7A1 (F). Elongation complexes stalled at + 21 (EC) were formed by addition of 100 μM ApU, 2 mM ATP, 10 μM GTP, 10 μM CTP and 10 μM 3'dUTP. 250 nM Mfd was added and samples were loaded on a gel at the time points indicated. (B) Time course of the displacement of stalled elongation complexes by Mfd at 37 °C. Elongation complexes were formed with wild-type RNAP (open triangles) and with RNAP βR529C (filled diamonds). The stability of RNAP βR529C elongation complexes was also monitored in the absence of Mfd (filled squares). Data points represent the percentage of elongation complex present at each time point, relative to the amount at t=0 and are expressed as the average of three independent experiments (with standard deviation).

Table 1*E. coli* K-12 derivatives used in these experiments.

Lab No.	Other No.	Genotype	Source
HL108	SR108	F- λ -, <i>thyA36</i> , <i>deoC2</i> , <i>IN(rrnD-rrnE)1</i>	J Cairns
HL961	MG1655	F- λ -, <i>rph-1</i>	C. Gross
HL978	CAG3008	F- λ -, <i>rph-1</i> , <i>rpoB3401</i> , <i>btuB::Tn10</i>	C. Gross
HL979		F- λ -, <i>rph-1</i> , <i>rpoB3401</i> , <i>btuB::Tn10</i>	C. Gross
HL1080		F- λ -, <i>rph-1</i> , <i>btuB::Tn10</i> , <i>rpoB(1552 AT->GC)</i>	Ding Jin
HL1082	DJ1017	F- λ -, <i>rph-1</i> , <i>cpsF4::Tn10</i> , <i>rpoB111</i>	Ding Jin
HL1126		F- λ -, <i>thyA36</i> , <i>deoC2</i> , <i>IN(rrnD-rrnE)1</i> , <i>btuB::Tn10</i>	this work
HL1128		F- λ -, <i>thyA36</i> , <i>deoC2</i> , <i>IN(rrnD-rrnE)1</i> , <i>btuB::Tn10</i> , <i>rpoB3401</i>	this work

Table 2

Mutations and amino acid changes in the strains used

Strain	rpoB allele	Nucleotide change	Amino acid change
HL108 (SR108)	wild type	none	none
HL961 (MG1655)	wild type	none	none
HL978	3401	1585 CG->TA	R529C
HL979	3401	1585 CG->TA	R529C
HL1080		1552 AT->GC	N518D
HL1082	111	1691 GC->AT	P564L
HL1126	wild type	none	none
HL1128	3401	1585 CG->TA	R529C

Table 3
Levels of β -galactosidase in *E. coli* derivatives tested for TCR. The values shown are pmols ONPG hydrolyzed/min/ 10^7 CFU, and are averages of values obtained from individual experiments.

Strain	RpoB change	+IPTG	-IPTG	Ratio	TCR
HL108	none	5756.	2.	2878.	+
HL1126	none	6579.	7.	1196.	+
HL961	none	2647.	15.	176.	±
HL1080	N518D	4071.	77.	53.	+
HL1082	P564L	2167.	16.	135.	+
HL978	R529C	2112.	7.	302.	+
HL979	R529C	605.	29.	21.	-
HL1128	R529C	7516.	16.	940.	-